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Docket No.: NEB-138-CIP

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EXAMPLE VI

Phosphoantibody to the Substrate Consensus Sequence for Akt: RXRXXT*

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The Akt protein kinase is an important regulator of cell survival and insulin signaling, but very few of its *in vivo* targets have been identified. Studies with synthetic peptide substrates of Akt (D.R. Alessi et al. FEBS Lett. 399:333-338 (1996)) as well as the analysis of known Akt phosphorylation sites on GSK-3 (T.F. Franke et al. Cell 88:435-437 (1997)), Bad (M. Pap et al. J. Biol. Chem. 273:19929-19932 (1998); Datta et al. Cell 91:231-241 (1997)), FKHR Brunet et al. Cell 96:857-868 (1999)), and Caspase-9 (M.H. Cardone et al. Science 282:1318-1321 (1998)) indicate that Akt phosphorylates its substrates only at a serine or threonine in a conserved motif characterized by arginine at positions -5 and -3.

To study and discover new Akt targets, an antibody was developed that specifically recognizes the phosphorylated form of the Akt substrate consensus sequence RXRXXT*. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr* represents phospho-threonine: Cys-X-X-X-Arg-X-Arg-X-X-Thr*-X-X-X-X. The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected

(SEQ ID NO: 45)

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was used, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr* represents phospho-threonine: Cys-X-X-X-X-X-Arg-Arg-X-Thr*X-X-X-X. The synthetic phospho-peptide was conjugated ^{to} KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

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Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Protein A eluate was first incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. This resin was then drained and the flow-through then incubated with phospho-peptide resin. This column was drained, washed twice with PBS, phospho-specific antibody eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

et al. EMBO J. 15:6541-6551 (1996)). RSK1 (Ser381) and the PKC's also contain this consensus site, phosphorylation of which is required for their activity (K.N. Dalby *et al.* J. Biol. Chem. 273:1496-1505 (1998); L.M. Keranen *et al.* Curr. Biol. 5:1395-
5 1403 (1995)).

To help study signaling pathways regulated by phosphorylation at these key regulatory sites we developed an antibody that detects phospho-serine and phospho-threonine only
10 when preceded by tyrosine, tryptophan or phenylalanine or when followed by phenylalanine. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Ser* or Thr* represents phospho-serine or
15 phospho-threonine: X-X-X-X-F-X-X-F-[S*/T*]-[F/Y]-X-X-X-X-C. This synthetic phospho-peptide was conjugated to KLH and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

(SEQ ID NO: 47)

20 Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A
25 chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from

Phospho-Akt Substrate Antibody

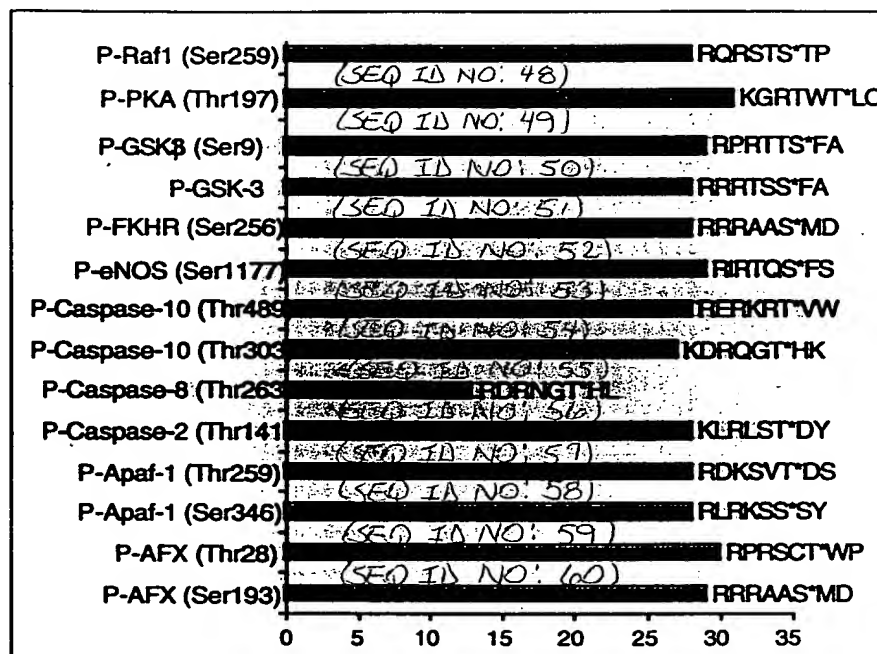
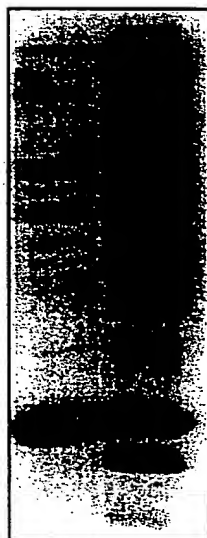


Figure 6: Signal to noise ratio of ELISA readings using Phospho-Akt Substrate Antibody with phospho-peptides of Akt substrates vs. non-phospho-peptides of Akt substrates.



- +
calyculin A

Figure 7: Western analysis of calyculin A-treated A431 cells using Phospho-Akt Substrate Antibody.

Phospho-PKA Substrate Antibody

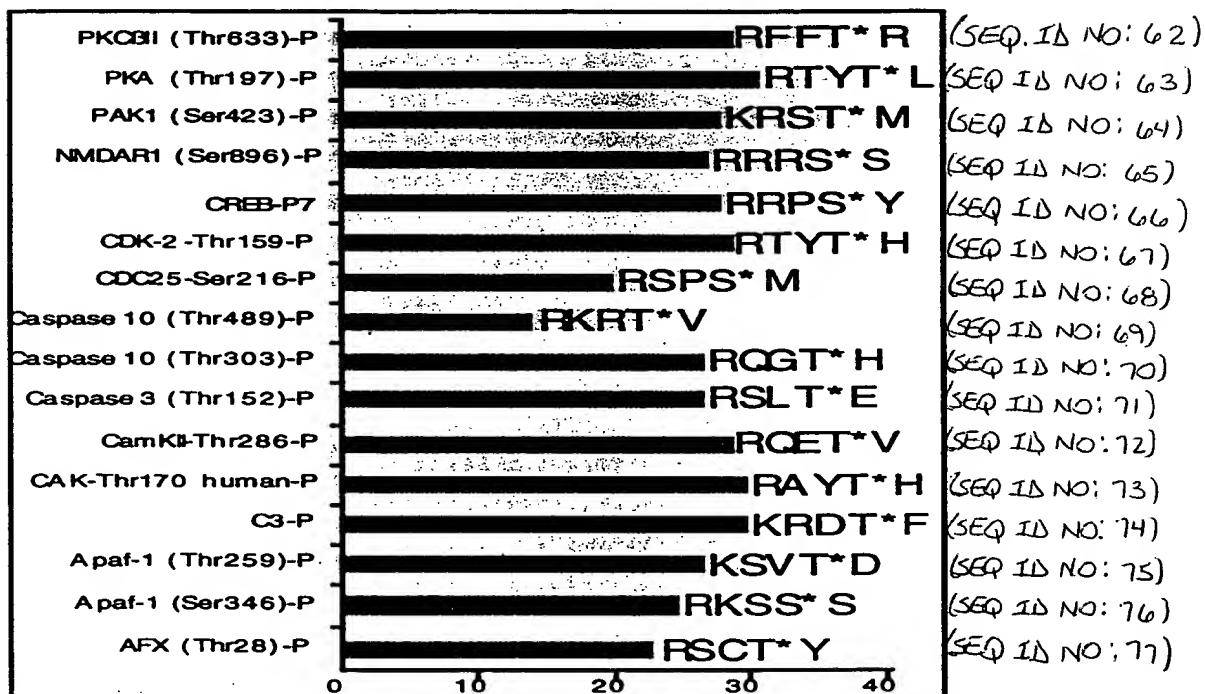


Figure 8: Signal to noise ratio of ELISA reading using phospho-PKA substrates antibody against peptides have Arginine or Lysine at -3 position.

Phospho-Serine/Threonine Phenylalanine Antibody

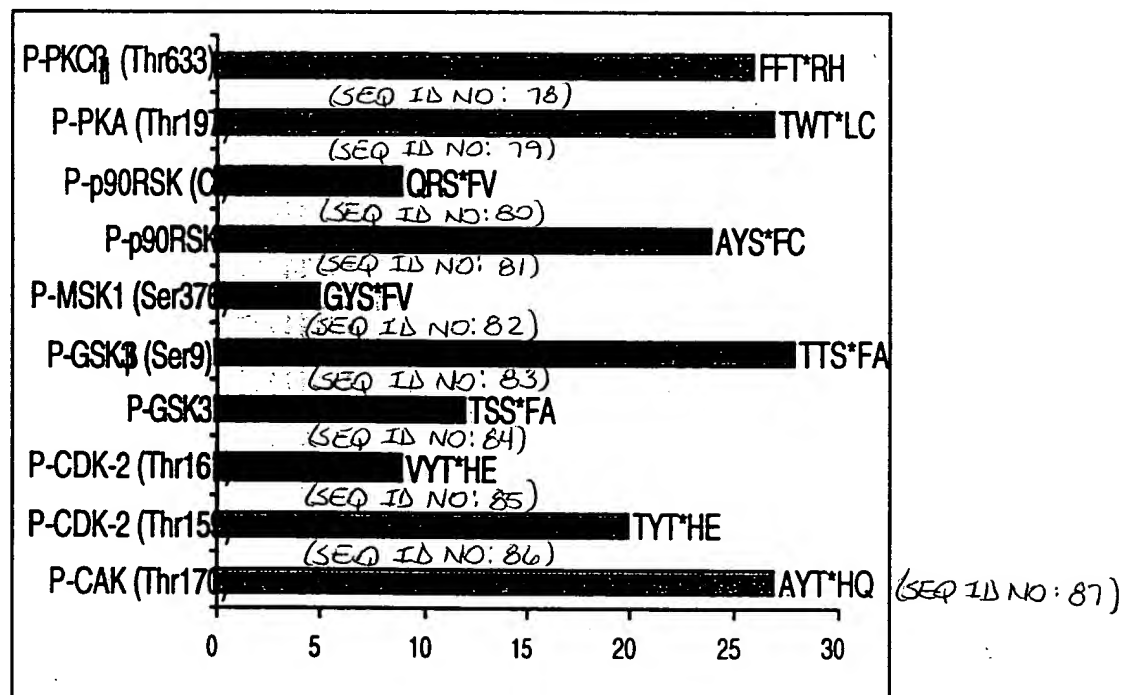


Figure 11: Signal to noise ratio of ELISA reading using phospho-Serine/threonine phenylalanine antibody against the peptides surrounded by phenylalanine, tyrosine or tryptophan.

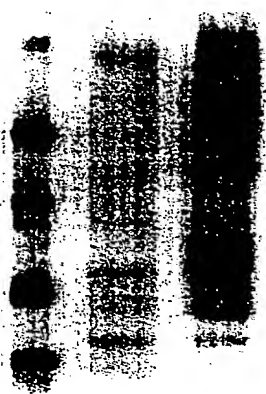


Figure 12: Western analysis of calyculin A-treated A431 cells using phospho-Serine/phenylalanine substates antibody.

calyculin A